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REGULAR ARTICLE

# Critical microcarrier properties affecting the expansion of undifferentiated human embryonic stem cells

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**Abstract** A variety of microcarriers may be used for the expansion of human embryonic stem cells (hESC) for cell therapy applications. This study investigated the effects of 10 types of microcarriers on hESC attachment efficiency, growth and pluripotency. High attachment efficiency was observed on uncoated microcarriers, however poor cell growth and/or gradual loss of pluripotency occurred during continuous passaging. Coating of the microcarriers with Matrigel resulted in higher cell yields and stable pluripotent states for at least three passages. Positively charged cylindrical cellulose microcarriers (DE52, DE53 and QA52) and large (190 μm) positively charged spherical microcarriers (Cytodex 1) exhibited high cell expansion potential and levels of pluripotency. Lower cell yields were obtained using smaller diameter spherical (65 μm and 10 μm) or macroporous beads. Instead of Matrigel, laminin coated microcarriers (DE53 and Cytodex 1) are capable of supporting the long term propagation and pluripotency of HES-2 and HES-3 cell lines. HES-2 cell line which was shown earlier to be shear resistant achieved similar cell growth and expression of pluripotent markers when cultured on both Matrigel (84% Tra-1-60, $1.43\times10^6$ cells/ml) and laminin (74% Tra-1-60, $1.37\times10^6$ cells/ml) coated microcarriers in spinner flasks. In contrast, HES-3 exhibited a decrease in cell yield, viability and pluripotent markers on laminin as compared with Matrigel coated microcarriers possibly due to shear sensitivity. Conventional microcarriers intended for propagation of mammalian cells are not suitable for long term propagation of hESC. Matrigel or laminin coating is essential for stable long term propagation of hESC on a variety of microcarriers.


## Introduction

Human embryonic stem cells (hESC) are considered to be a renewable source for cell therapy due to their ability to differentiate into multi-lineage cell types of the three primary germ layers (Reubinoff et al., 2000; Thomson et al., 1998). Ever since their first derivation, hESC have always been cultured as dense colonies which were mechanically dissected and transferred from one tissue culture dish to another

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(Reubinoff et al., 2000). Either mouse or human feeder cells have been used to maintain the undifferentiated state of hESC *in vitro* (Rao and Zandstra, 2005). To simplify the cultivation protocol, the development of feeder free methods using extracellular matrices (ECM) as surface coatings, such as Matrigel and other ECMs proteins such as laminin, vitronectin and fibronectin have been developed (Skottman and Hovatta, 2006; Braam et al., 2008). In these systems, conditioned media from feeder cells or defined media supplemented with growth factors were used (Skottman and Hovatta, 2006; Chin et al., 2007). In order to facilitate the expansion of hESC, several studies have demonstrated the use of cell dissociation enzymes to generate single cells for passaging (Hasegawa et al., 2006; Ellerström et al., 2007; Bajpai et al., 2008). The expansion of undifferentiated stem cells has always been carried out manually on 2-dimensional (2D) platforms. A study by Veraitch et al. (2008) showed that manual processing has inherent problems of inconsistent cell proliferation and phenotypic variability. Recently, an automated process handling multiple tissue culture flasks has been demonstrated to replace manual passaging of hESC to improve consistency (Thomas et al., 2009).

While the cultivation of hESC on microcarriers is relatively novel in the field of stem cells, the use of microcarriers for cell culture in the pharmaceutical industry is a well established technology. To design cell-compatible microcarriers, researchers have comprehensively investigated the effect of the microcarrier properties on the attachment and growth of various cell types. The requirements and properties of optimal microcarrier for mammalian cell culture were reviewed by Reuveny (1990). Generally such microcarriers have functional attachment groups on their surface (for example, positive charge of about 1.5–2.5 meq/g dry material, collagen or gelatin) and a buoyant density of between 1.03 and 1.10 in order to allow microcarrier suspension by slow agitation. Spherical morphology with dimension between 100 and 250 μm allows the propagation of hundreds of cells on each bead, and they are designed with different porosity levels (microporous and macroporous) (Reuveny, 1990). A variety of dextran, cellulose, polystyrene, glass or gelatin microcarriers with different functional groups, shapes and sizes are produced commercially (Reuveny, 1990; Malda and Frondoza, 2006) or they can also be obtained or derived from available chromatography matrices (Reuveny et al., 1980, 1983). Recently, several groups have demonstrated that culturing undifferentiated hESC on microcarriers is more robust and feasible for scale-up than 2-dimensional (2D) cultures (Phillips et al., 2008; Oh et al., 2009; Nie et al., 2009; Storm et al., 2010; Kehoe et al., 2010). hESC can be propagated for many passages without losing pluripotency potential as shown by the spontaneous differentiation of embryoid bodies (Oh et al., 2009; Nie et al., 2009). These microcarrier cultures can be scaled up in conventional controlled stirred tank bioreactors (Chu and Robinson, 2001) and expanded *in situ* without the need for enzymatic treatment (Oh et al., 2009), thus simplifying the process of culture expansion. Furthermore, subsequent differentiation of hESC on microcarriers *in situ* to a particular lineage of choice has been demonstrated for cardiomyocytes (Phillips et al., 2008; Oh et al., 2009; Lecina et al., 2010), definitive endoderm (Lock and Tzanakakis, 2009), neuronal and pancreatic cells (Phillips et al., 2008).

Selecting suitable microcarriers for cell propagation is usually the first step for the development of any microcarrier based process including hESC culture. Different commercially available microcarriers were used by Phillips et al. (2008), Oh et al. (2009), Nie et al. (2009), and Lock and Tzanakakis (2009). These were tested with different hESC lines for short term (8 days (Lock and Tzanakakis, 2009)) and long term (10 (Nie et al., 2009) to 25 passages (Oh et al., 2009)) propagation. From these studies, it is difficult to determine the effect of microcarrier properties on hESC growth and pluripotency. Hence, there is a need for a comparative evaluation of microcarriers with different properties for their effects on hESC growth and pluripotency.

In order to evaluate the correlation between microcarrier properties and long term hESC growth, we investigated 10 different microcarriers and 7 ECM coatings for their effect on cell attachment efficiencies, long term maintenance, and expansion of undifferentiated hESC. It was found that a variety of Matrigel or laminin coated microcarriers can support the long term maintenance of pluripotent cells. Finally, the expansion of two hESC lines on laminin coated microcarriers in spinner cultures was successfully demonstrated.

## Results

### Comparison of HES-3 attachment and growth on different uncoated microcarriers

The cell attachment efficiency, the consistency of cell growth and percentage of cells expressing pluripotency marker Tra-1-60 on the microcarriers are shown in Fig. 1. Tra-1-60 is selected since it was found to be a sensitive marker for onset of differentiation in hESC cultures (Leung et al., 2010; Yap et al., 2011; Chin et al., 2010).

Fig. 1A shows that after 2 h, significant cell attachment (over 60%) was observed on the positively charged microcarriers (DE53, QA52, DE52, Cytodex 1, Tosoh 65 PR, Cytopore 2). The attachment was not affected by the type of matrix (cellulose and dextran), shapes (cylindrical or spherical), size (diameter 65-250 μm), porosity (microporous or macroporous) and type of positive charge (tertiary, quaternary amine, or derivatized with positively charged protein, protamine). Lower levels of cell attachment (38%) were observed on small diameter (10 μm) protamine derivatized positively charged beads (Tosoh10 PR), probably since these beads, which are smaller than the cells, do not allow for cell attachment and spreading but rather generate compact aggregates.

Collagen coated microcarrier (Cytodex 3) showed high cell attachment efficiency (77%), similar to positively charged cellulose microcarriers. The macroporous gelatin microcarriers (Cultispher G) showed low attachment efficiency (23 ± 8%). As expected, very low cell attachment was observed on the negatively charged microcarriers (CM-52) or the negatively charged control tissue culture polystyrene 6-well plate.

Most of the microcarriers listed in Table 1, with the exception of negatively charged CM52 microcarrier, were able to support cell growth and pluripotency for two passages after seeding from 2D monolayer culture (results not shown). However, at passage 3 we observed a wide range of cell yields between microcarriers ($0.9\times10^5$ to $9.2\times10^5$ cells/ml), cystic structures (similar to those previously reported (Oh et al.,

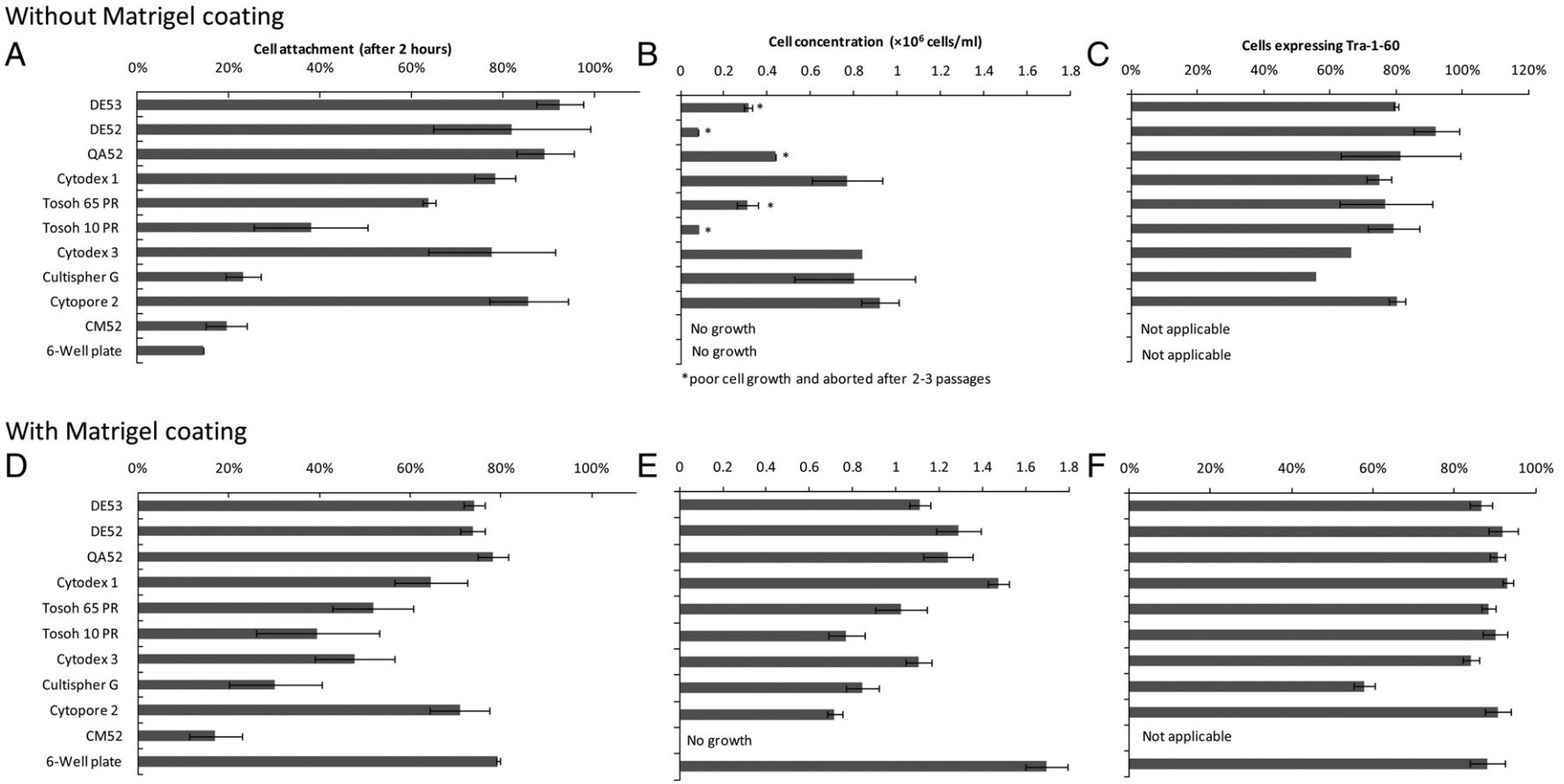



**Figure 1** HES-3 cell attachment, growth and pluripotency on a variety of non-coated (A–C) and Matrigel coated (D–F) microcarriers. (A, D) cell attachment efficiency (%) after 2 h in culture. (B, E) cell concentration on day 7 at passage 3 or later. (C, F) percentages of cells expressing pluripotent marker Tra-1-60 at passage 3 or later. $1.6 \times 10^5$ cells were seeded on microcarriers at concentrations given in Table 1. For non-coated microcarriers, cells were cultured for at least two consecutive passages. For coated microcarriers, cells were propagated on DE53 for 17 passages, DE52 for 3 passages, QA52 for 3 passage, Cytodex 1 for 11 passages, Cytodex 3 for 8 passages, Tosoh 65 PR for 10 passages, Tosoh 10 PR for 10 passages and Cytopore 2 for 5 passages. Results indicate the average values obtained from all runs. Error bars indicate the standard error.

2009)) and only 53 to 85% of the cells expressed Tra-1-60 (Figs. 1B and C). The best hESC growth was observed on the 4 large spherical microcarriers with comparable cell growth ($7.7\times10^5$ to $9.2\times10^5$ cells/ml) with 67–85% of cells expressing Tra-1-60 (Figs. 1B and C). Upon continued passaging of these cultures, further decreases in Tra-1-60 expression were observed (data not shown).

## Long term growth and pluripotency of hESC are improved when microcarriers are coated with Matrigel

On coating with Matrigel, most of the 10 types of microcarriers show a decrease in cell attachment efficiency (Fig. 1D). For example, positively charged DE53 and QA52 show significant decreases in cell attachment, 11% and 18% respectively (p-value $<0.05$). The level of decrease probably depends on the type and level of positive charge. Collagen coated Cytodex 3 microcarriers showed a 30% (p-value $=0.035<0.05$) decrease in cell attachment. The reduction in cell attachment efficiency can be attributed to the Matrigel coating which masks the positive charge or collagen coating of the microcarriers. Small Tosoh10 PR beads remained unfavorable for cell attachment. The negatively charged microcarrier (CM52) once again generated the lowest cell attachment.

On the other hand, Matrigel coating had a profound improvement on cell yields and pluripotency in long term cultures (Figs. 1E, F and Supplementary Fig. 1). Cell yields of $8\times10^5$ to $1.5\times10^6$ cells/ml are significantly higher by 1.9 to 18 fold than that obtained with uncoated microcarriers, except for Cytopore 2 which showed no improvement (Fig. 1E); e.g. Cytodex 1 has 1.9 fold improvement with p-value ($1.01\times10^{-5}<0.05$). No major cell necrosis or cell death was observed in the static microcarrier cultures. Immunohistology analyses show that cells in the center of the aggregates appear intact without major membrane damage (Supplementary Fig. 1). Moreover cells harvested from this aggregated stationary cultures had more than 90% viability as estimated by viable trypan-blue staining (data not shown).

Most importantly, the majority of the hESC microcarrier cultures were able to maintain the expression of Tra-1-60 above 80% for 3 to 11 passages. Immuno-staining of HES-3 cultured on Matrigel coated microcarriers (DE53, Cytodex 1 and Tosoh 65 PR) showed positive staining for Oct4 and Tra-1-60 (Supplementary Fig. 1). The only microcarrier that caused a loss of pluripotency is Cultispher G with Tra-1-60 expression decreasing from 86% to 53% after the second passage and maintained at $58\pm5\%$ for the subsequent 4 passages (Fig. 1F). It seems that the gelatin surface of this microcarrier has a negative effect on pluripotency.

Microcarrier shape and size affect cell-microcarrier aggregate morphology as shown in Fig. 2. The cylindrical cellulose DE53 formed compact cell-microcarrier aggregates after 5 days of cultivation. Cytodex 1 generates more open aggregate structures with thinner cell layers adhering onto strings of large (190 μm) microcarriers, whereas the smaller 65 μm Tosoh65 PR produced even more compact cell-microcarrier aggregates. Very dense aggregates were formed on 10 μm beads which are smaller in size than the cells (Tosoh10 PR, Fig. 2). These condensed structures might have contributed to lower cell yields.

In light of the above findings, we chose to continue with cylindrical and spherical positively charged microcarriers (DE53 and Cytodex 1), which showed robust cell attachment, growth and maintained pluripotency for at least 10 passages as shown in Fig. 3. Closer examination of cell morphology by scanning electron microscopy further illustrates the ability of hESC to grow in aggregates on the cylindrical DE53 and spherical Cytodex 1 microcarriers. The more compact structured cell-DE53 microcarriers might have an advantage over Cytodex 1, perhaps tolerating higher shear stress rates in a stirred bioreactor (Reuveny, 1990).

## Screening for a defined source of extracellular matrix (ECM) to support hESC attachment and growth on microcarriers

While it was clear that Matrigel coating improved growth of hESC for most of the tested microcarriers, Matrigel is considered as an undefined source of ECM which comprised primarily of laminin, collagen IV, and entactin as well as several other components such as heparan sulfate proteoglycans (Kleinman et al., 1982; Bissell et al., 1987). In order to replace Matrigel with a defined ECM, we have evaluated hyaluronic acid (from bovine and Streptococcus), heparan (from bovine and porcine), vitronectin, fibronectin and laminin coatings. As shown in Fig. 4A, laminin coated microcarriers achieved the highest cell yield in 7 days of culturing compared to all other coatings except Matrigel. Although fibronectin- and vitronectin-coatings can replace Matrigel for hESC growth in 2D tissue culture plates (data not shown), we observed reduced cell growth when they were coated on the microcarriers. As shown in Fig. 4B, the morphology of HES-3 cultured on laminin-coated DE53 microcarriers was similar to those cultured on Matrigel coated ones.

## hESC maintained growth and remained pluripotent when cultured on laminin-coated microcarriers

Encouraged by the expansion capability and stable pluripotency achieved by the laminin coated microcarriers, we continued the studies in long term culture to examine its effects on cell growth, pluripotency and karyotype stability. We have carried out six consecutive passages using two hESC lines, HES-3 and HES-2 to monitor cell yield and expression of pluripotent markers. As seen from Fig. 5A, HES-3 on laminin coated DE53 generated a comparable cell yield ($10.1\pm2.4\times10^5$ cells/ml) to Matrigel coated ones ($11.0\pm2.4\times10^5$ cells/ml) (p-value $=0.14$, $n=7$). Similar observations of cell yields was seen for HES-2 with Matrigel coated DE53 ($10.9\pm3.4\times10^5$ cells/ml) versus laminin coated ones ($8.8\pm3.3\times10^5$, p-value $=0.053$, $n=7$) (Fig. 5C). As for pluripotency, comparable percentage of cells expressing mAb84 (~85–96%) or Tra-1-60 (~88–97%) were obtained for both cell lines when compared to those grown on Matrigel-coated DE53 microcarriers (Figs. 5A and C). Immuno-staining of HES-3 cultured on laminin coated DE53 microcarriers show positive staining of Oct4 and Tra-1-60 (Supplementary Fig. 1).

However, growth of HES-3 on laminin coated Cytodex 1 generated lower average cell yields than Matrigel

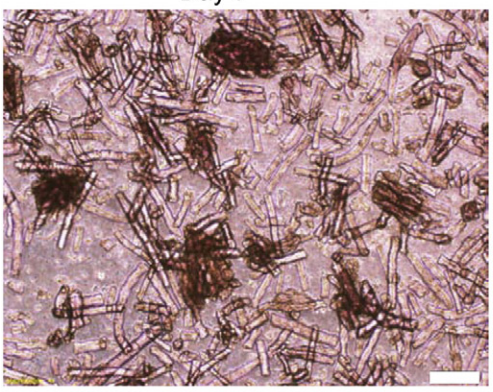
**Table 1** Characteristics of microcarriers used in this study.

| Microcarrier | Manufacturer | Shape | Dimension | Surface area[#] | Matrix | Surface feature | Charge density | Coatinglcoupling | Concentration in culture |
|---|---|---|---|---|---|---|---|---|---|
| DE53 | Whatman | Cylindrical | L 130 ± 60 μm × D 35 ± 7 μm | 6.8cm$^2$/mg[a] | Cellulose | Diethylaminoethyl (Tertiary amine) | 1.8–2.2 meq/g dry weight* | Not applied | 4 mg/ml |
| DE52 | Whatman | Cylindrical | L 130 ± 60 μm × D 35 ± 7 μm | 6.8cm$^2$/mg[a] | Cellulose | Diethylaminoethyl (Tertiary amine) | 0.88–1.08 meq/g dry weight* | Not applied | 4 mg/ml |
| QA52 | Whatman | Cylindrical | L 130 ± 60 μm × D 35 ± 7 μm | 6.8cm$^2$/mg[a] | Cellulose | Quaternary Ammonium | 1.09 meq/g dry weight* | Not applied | 4 mg/ml |
| CM52 | Whatman | Cylindrical | L 130 ± 60 μm × D 35 ± 7 μm | 6.8cm$^2$/mg[a] | Cellulose | Carboxymethyl | 1.0 meq/g dry weight* | Not applied | 4 mg/ml |
| Cytodex 1 | GE Healthcare | Spherical | 190 ± 58 μm | 4.4 cm/mg[b] | Cross-linked dextran | Diethylaminoethyl (Tertiary amine) | 1.4–1.6 meq/g dry weight* | Not applied | 1 mg/ml |
| Cytodex 3 | GE Healthcare | Spherical microporous | 175 ± 36 μm | 2.7cm$^2$/mg[b] | Cross-linked dextran | Denatured collagen | Not available | 16 mg denatured type 1 collagen/ cm$^2$ | 1 mg/ml |
| Cultispher G | Hyclone | Spherical macroporous | 255 ± 125 μm (Pore size 10–20 μm) | 40cm$^2$/mg[b] | Cross linked gelatin | Gelatin | Not available | Not applied | 1 mg/ml |
| Cytopore 2 | GE Healthcare | Spherical macroporous | 240 ± 40 μm (Pore size 30 μm) | 11cm$^2$/mg[b] | Cross-linked cotton cellulose | Diethylaminoethyl (Tertiary amine) | 1.65–1.95 meq/g dry weight | Not applied | 1 mg/ml |
| Toyopearl AF-Tresyl-650 M (Tosoh 65PR) | Tosoh Bioscience | Spherical | 65 ± 25 μm | 4.2cm$^2$/mg[a] | Hydroxylated methacrylate | Tresyl ligand derivatized with Protamine sulfate (Primary amine) | No data | 9.6×10$^2$ mg protamine sulfate/ mg dry beads | 1 mg/ml |
| TSKge1 Tresyl-5PW (Tosoh 10 PR) | Tosoh Bioscience | Spherical | 10 μm | 9.0cm$^2$/mg[a] | Hydroxylated methacrylate | Tresyl ligand derivatized with Protamine sulfate (Primary amine) | No data | 9.6×10 mg protamine sulfate/ dry mg beads | 0.2 mg/ml |

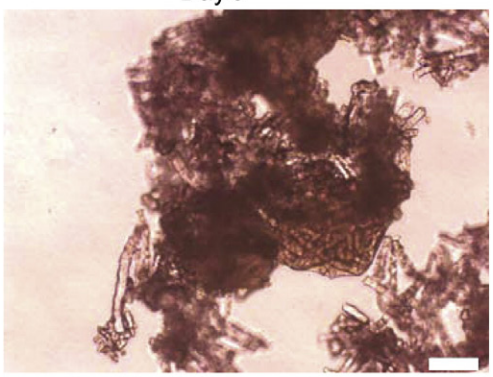
*Small ion exchange capacity; [#]surface area per milligram dry weight; [a]experimental measurement; [b]provided by manufacturer.

**DE53**

Day 1

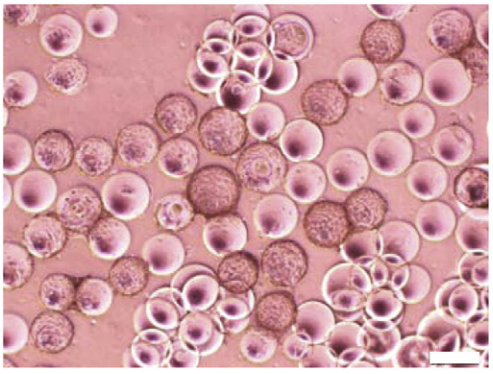


Day 5



**Cytodex 1**

**Day 1**



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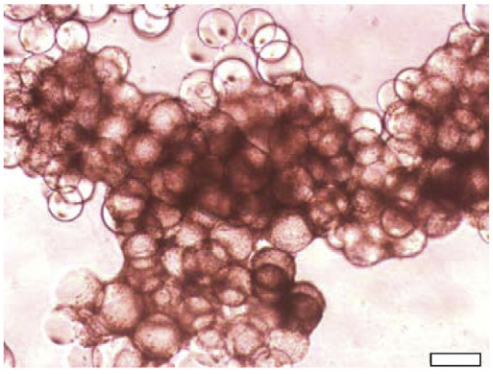
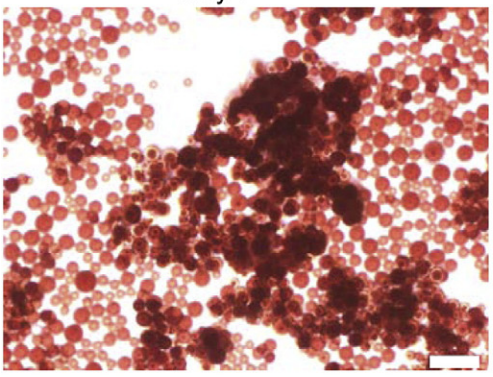


**Tosoh 65 PR**

Day 1



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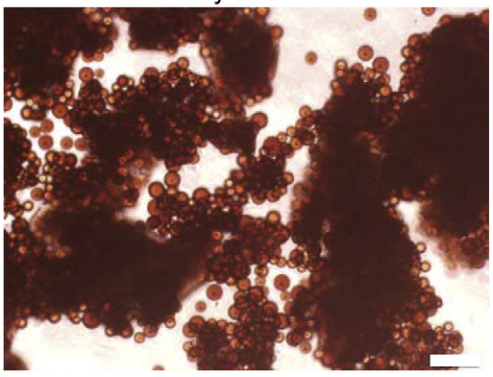
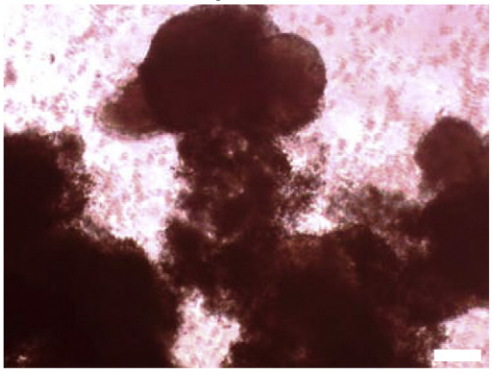


**Tosoh 10 PR**

Day 1

Day 5



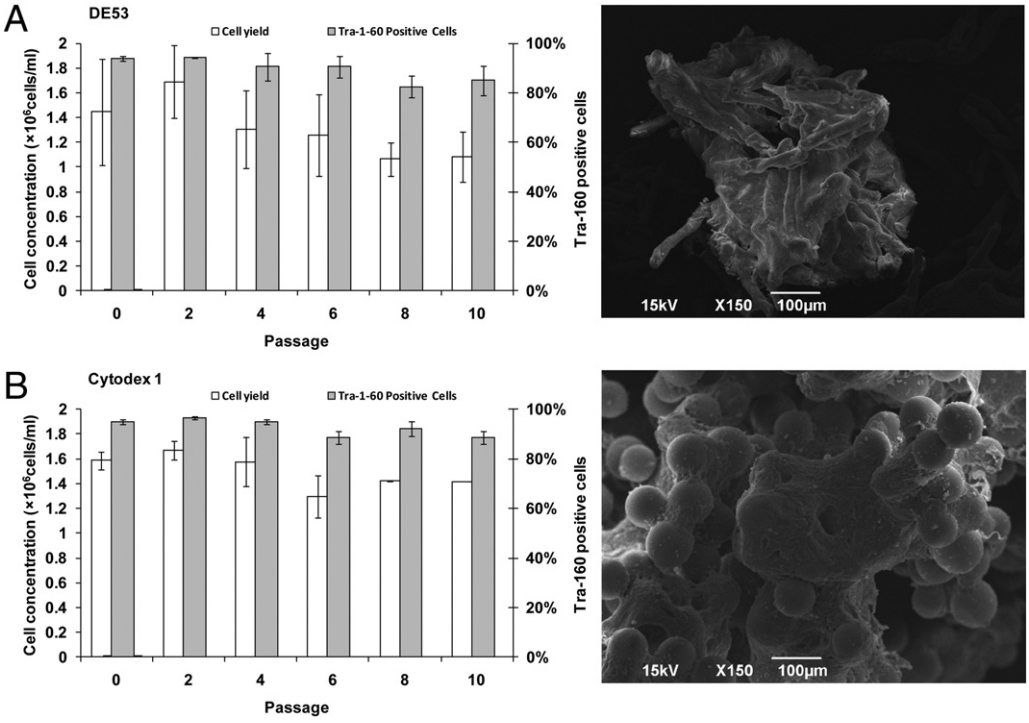



**Figure 3** Long term growth and pluripotency on Matrigel coated DE53 (A) and Cytodex 1 (B) microcarriers (10 passages). In each passage $0.8\times10^5$ cells/ml were seeded on 1 mg/ml microcarriers for 7 days. Cell concentration (white bars) and Tra-1-60 expression (gray bars) were measured on day 7. Error bars indicates standard error. SEM micrographs of HES-3 on DE53 and Cytodex 1 were taken from 7 day old cultures.

coated Cytodex 1 ($9.4\pm1.1\times10^5$ versus $14.7\pm1.4\times10^5$, p-value $=6.5\times10^{-6}<0.05$, n=7). The percentage of cells expressing Tra-1-60 was reduced after the second passage (from 95% to 83%) and remained ~80% for the subsequent passages (Fig. 5B). Flow cytometry histograms of HES-3 cultured on laminin coated Cytodex 1 expressing pluripotent markers (Oct4, TRA-1-60 and mAb84) after passages 6 are shown in Supplementary Fig. 2.

To confirm pluripotency, hESC from laminin-coated DE53 microcarriers (passage 6) were differentiated by both EBs generation and teratoma formation in SCID mice. Fig. 6A show cells stained positive for representative markers alpha-fetoprotein, AFP (Endoderm), β-III tubulin (Ectoderm) and smooth muscle actin, SMA (mesoderm). The increased expression of representative genes from the endoderm, mesoderm, and ectoderm lineages and decrease in Oct4 and Nanog was also observed (Fig. 6B). Furthermore, stable karyotype was maintained for at least 10 passages (Fig. 6C) and teratoma formed in SCID mice generated tissues from the three germ lineages, namely rosettes of neural epithelium, gut-like epithelium and cartilage (Fig. 6D).

## Expansion of hESC on laminin-coated microcarriers in spinner flask

In order to test the scale-up potential, we compared the growth of HES-2 (passage 8) and HES-3 (passage 4) on laminin-coated microcarriers to those on Matrigel-coated ones in spinner flasks. Fig. 7A shows that shear resistant HES-2 cells (Leung et al., 2010) exhibited comparable cell growth on both laminin- and Matrigel-coated microcarriers, with a cell yield of about $1.4\times10^6$ cells/ml on day 7, maintenance of high cell viability above 81% and similar percentages of cells expressing Tra-1-60 and mAb84 pluripotency markers. On the other hand, the shear sensitive HES-3 cell line (Leung et al., 2010) exhibited reduced cell growth, viability and pluripotency when propagated on laminin coated microcarriers as compared to the Matrigel coated ones. Cell yields at day 7 dropped from $3.42\times10^6$ to $1.90\times10^6$ cells/ml, with much lower cell viabilities throughout the culture and pluripotent markers decreased to very low levels (Figs. 7B and C). It appears that Matrigel coating with its gelatinous nature protects to some degree the HES-3 cells from mechanical stress that induces these cells to differentiate (Leung et al., 2010).

## Discussion

Microcarrier technology was introduced by van Wezel (1967) in 1967. Since then this technology has been used in a wide variety of biological applications, especially vaccines production from anchorage dependent cell lines (Chu and Robinson, 2001). The requirements and properties of optimal microcarrier for mammalian cell culture were reviewed by

**Figure 2** Phase contrast images of HES-3 cells cultured on Matrigel coated DE53, Cytodex 1, Tosoh 65PR and Tosoh 10PR microcarriers. Scale bars indicate 200 μm.

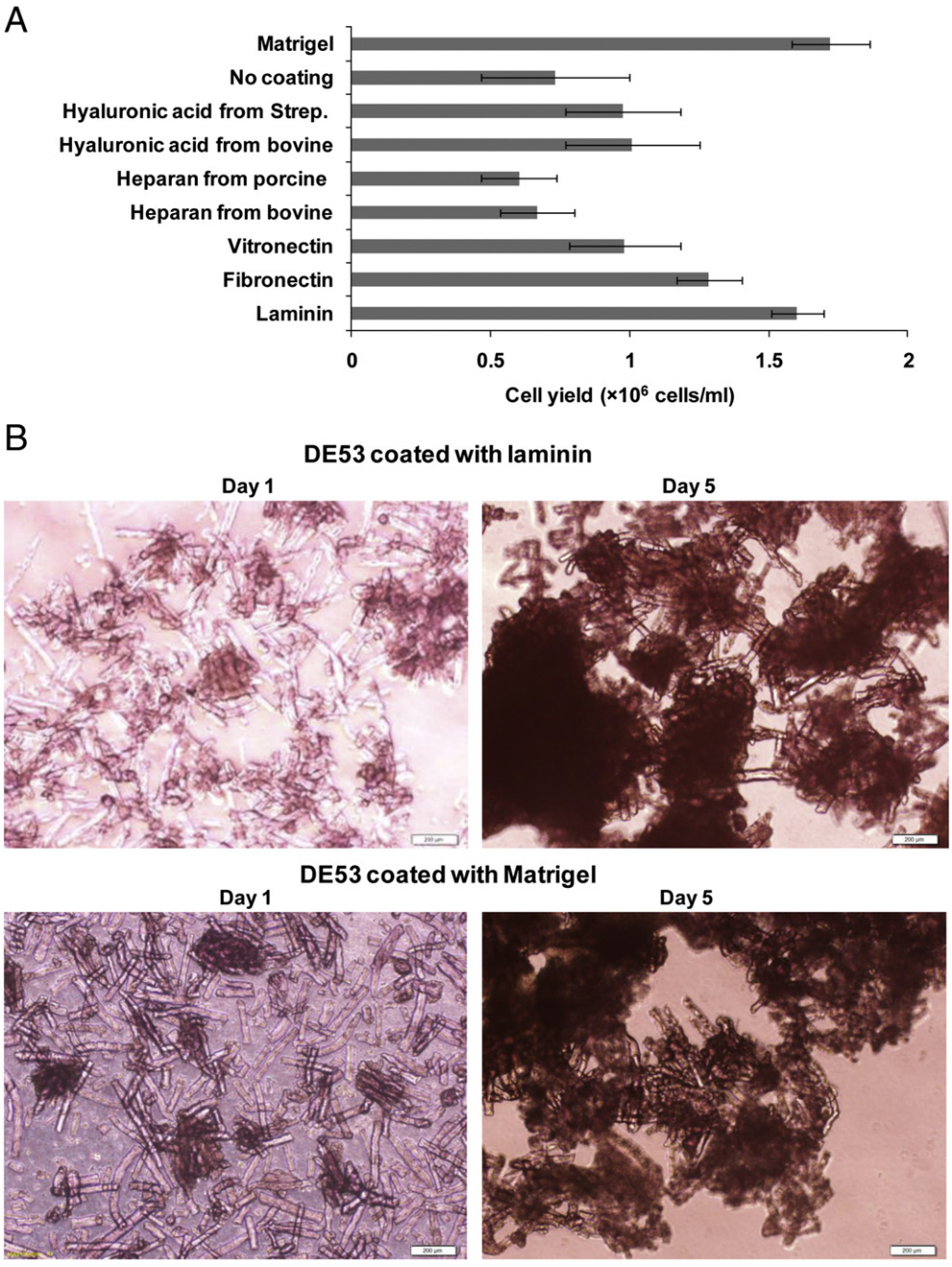



**Figure 4** HES-3 propagation on cellulose microcarriers (DE53) coated with different ECM components (A) $1.6\times10^5$ cells/ml were seeded on 4 mg/ml DE53 microcarriers, after two passages cell fold expansion was determined. Error bars indicates standard error. (B) Phase contrast images of cells cultured on DE53 coated with laminin and Matrigel. Scale bars indicate 200 μm.

Reuveny (1990). A variety of microcarriers having different functional attachment groups, matrices, shapes and porosity are available commercially or can be obtained or derivatized from chromatography matrices (Table 1) (Reuveny et al., 1980, 1983).

Translating stem cell research to clinical applications relies on the scaling-up of undifferentiated stem cell cultures and their subsequent differentiation into end products that meet quality and quantity requirements. An estimate of the amount of hESC required for clinical applications was calculated in recent review by Mummery (2005). The author suggested that one would need $5\times10^9$ undifferentiated hESC to repair an infracted heart. The need for this large amount of cells provides an opportunity to microcarrier technology for the large scale propagation of undifferentiated hESC in stirred reactors. For this purpose we have examined 10 types of microcarriers (Table 1) for their ability to support long term propagation of hESC. Our findings revealed that these microcarriers are not optimal for hESC culturing. Although cells attached efficiently to most of the microcarriers (up to 92% within 2 h with cellulose microcarriers DE52, QA52 and DE53), in long term propagation they exhibited poor cell yields and cells have a tendency to differentiate. The best microcarriers were Cytodex 3 and Cytopore 2 which have 78%

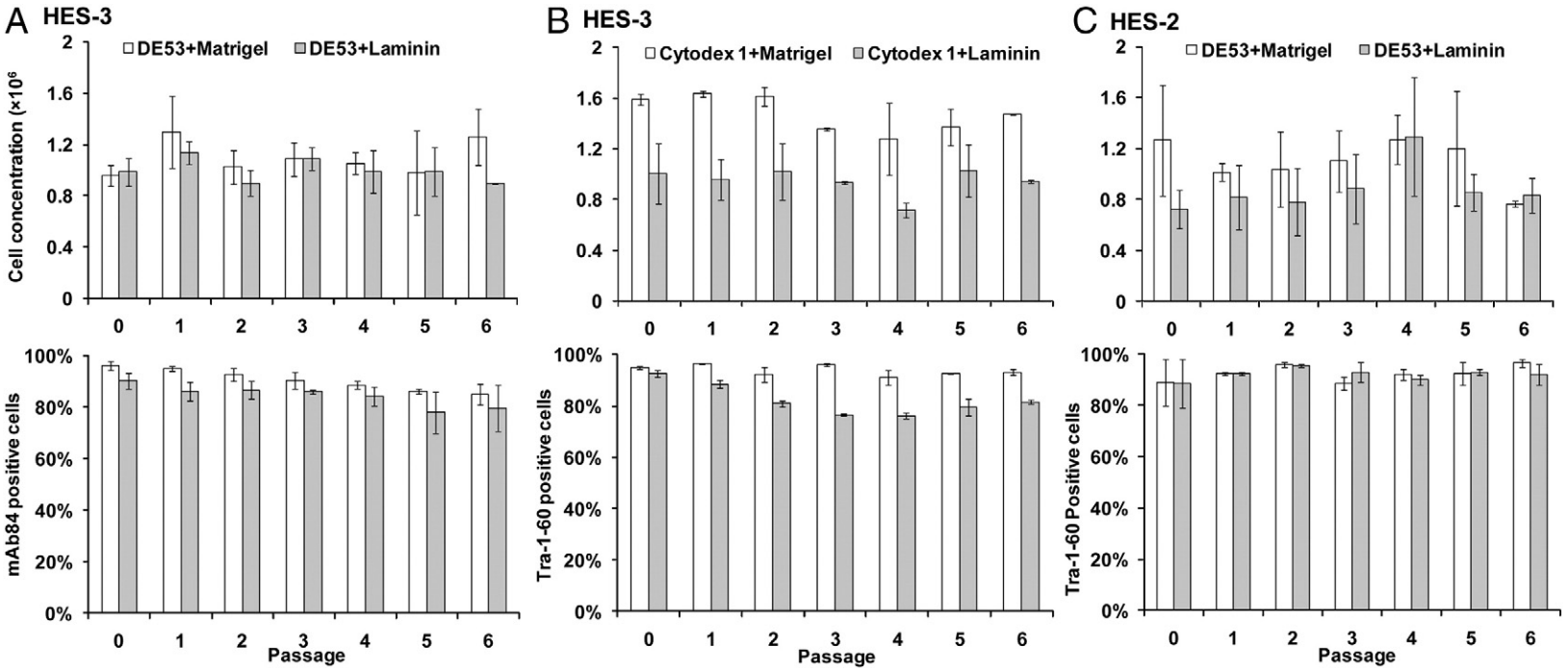



**Figure 5** Propagation of hESC in static conditions on Matrigel (white bars) versus laminin (gray bars) coated microcarriers. (A) HES-3 on DE53 microcarriers (B) HES-3 on Cytodex 1 microcarriers and (C) HES-2 on DE53 microcarriers. $0.8\times10^5$ cells/ml were seeded on 4 mg/ml microcarriers of DE53 or 1 mg/ml of Cytodex 1 and cultured for 6 passages. At day 7 of each passage cell concentration and percentages of cells expressing pluripotent markers were determined. Error bars indicates standard error.

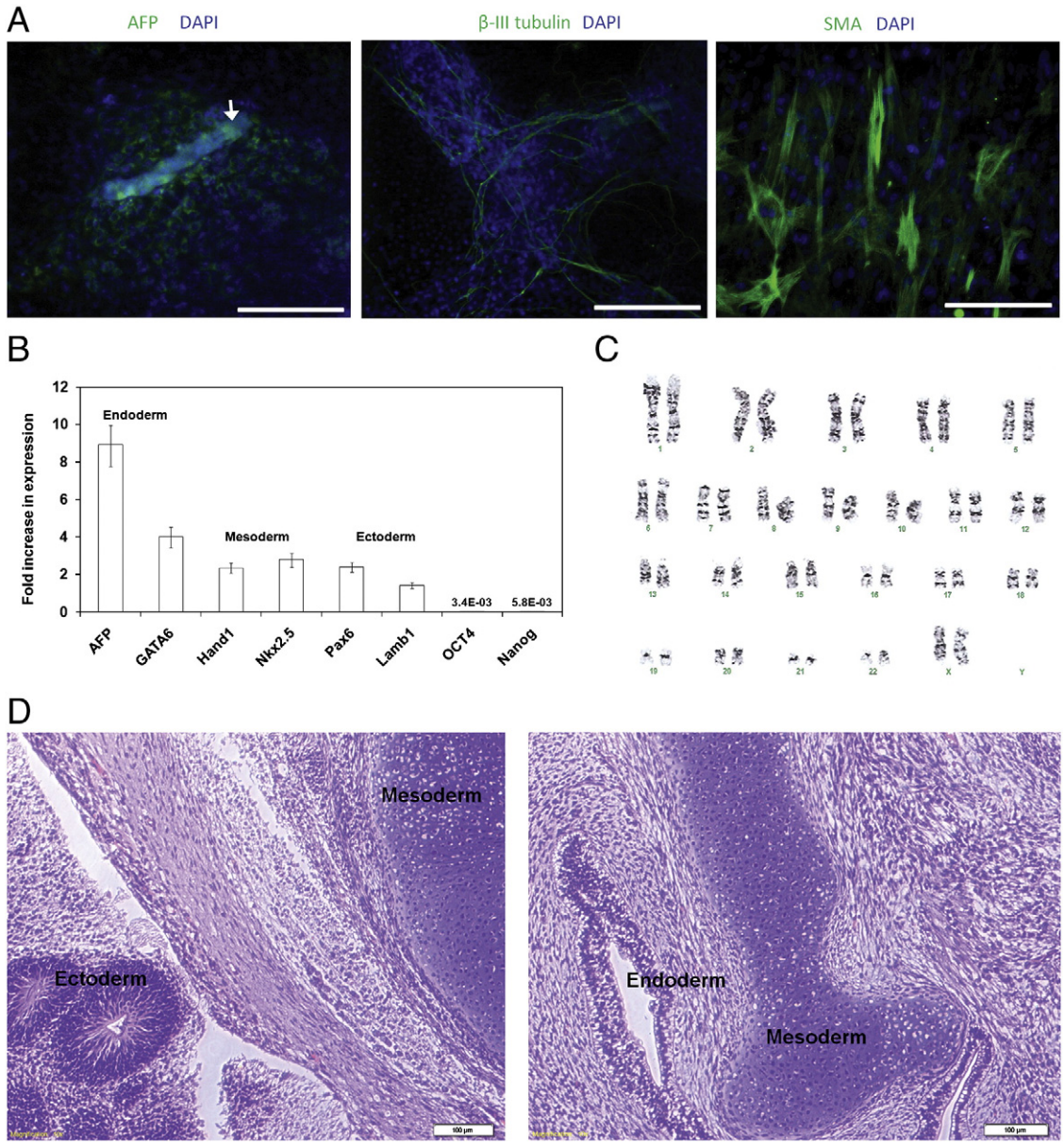
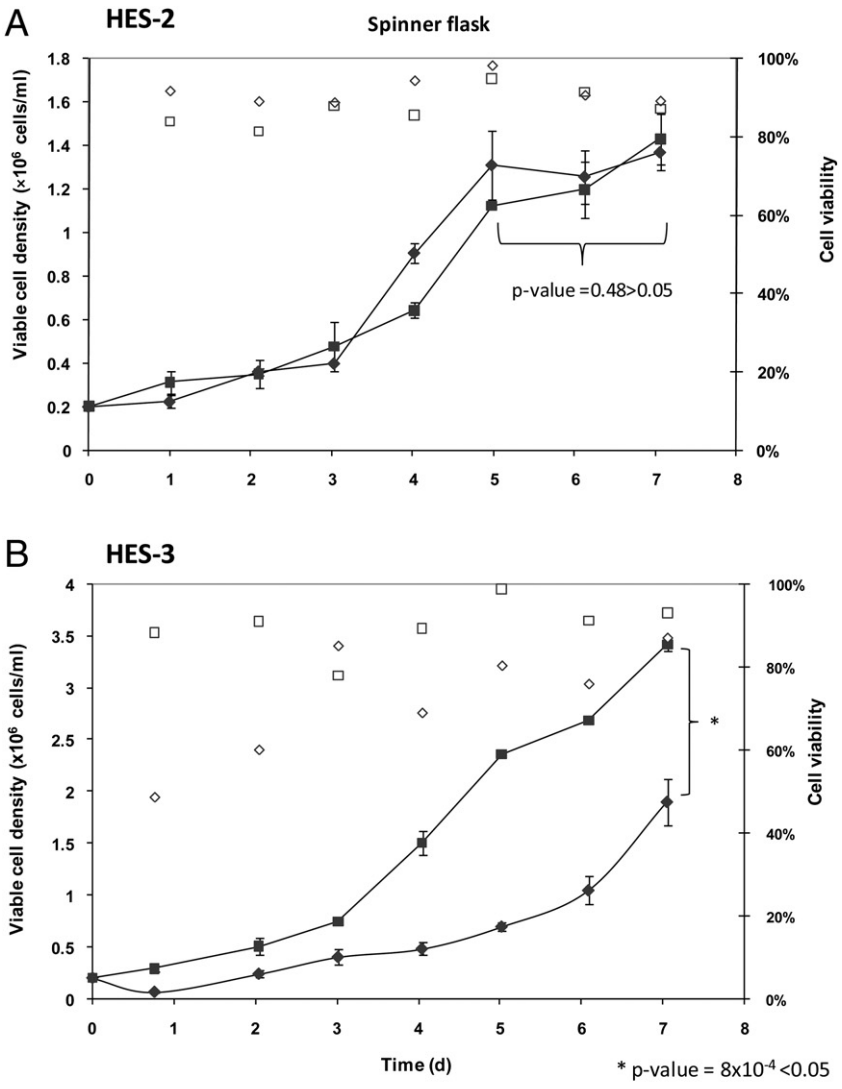



**Figure 6** Spontaneous differentiation of HES-3 cultured on laminin coated DE53 microcarriers (A) Immuno staining showing the formation of cells expressing AFP (endoderm), β-III tubulin (ectoderm) and SMA (mesoderm). A cylindrical DE53 microcarrier is surrounded by cells expressing AFP. Arrow indicates the autofluorescence of DE53 microcarrier (B) Quantitative real time PCR showing up regulation of genes associated with the formation of three germ layers. (C) Diploid karyotype of HES-3 after 10 passages. (D) Hematoxylin-eosin staining of teratoma generated in SCID mouse showing the three germ layers, neural rosettes (ectoderm), gut epithelia (endoderm) and cartilage (mesoderm). Scale bar indicates 200 μm.

and 85% attachment efficiency (2 h) and relative high cell yield of $8.4\times10^5$ and $8.0\times10^5$ cells/ml at day 7. These results are in agreement with Nie et al. (2009) who screened several commercial microcarriers using H1 and H9 hESC and found that Cytodex 3 was the most suitable microcarriers for cell attachment and viability. On the other hand Phillips et al. (2008) found that other cell line,ESI-017, can grow for 6 passages on Solohill Hillex II (spherical microcarrier having polystyrene matrix derivatized by cationic trimethyl ammonium) and not on other commercial microcarriers due to poor cell attachment (Cytodex 3, Solohill Plastic+, Solohill Pronectin, MicroHex, and Cytodex 1). In summary, these results show that commercially available mammalian cell microcarriers are not optimized for the long term propagation of hESC.

Coating of the microcarriers with ECM matrix (Matrigel) resulted in improved hESC growth. Matrigel, which contains mainly laminin, collagen IV, entactin and heparan sulfate proteoglycans (Kleinman et al., 1982; Bissell et al., 1987), binds to the microcarrier surface, generating a thin layer of coating which can be observed microscopically with fluorescence imaging of anti-laminin staining as shown in the study by Nie et al. (2009). Matrigel coating of the microcarrier can reduce cell attachment efficiency in most of the tested




C

| hESC line | Coating on DE53 | Tra-1-60 positive cells | mAb84 positive cells |
|---|---|---|---|
| HES-2 | Matrigel | 87% | 84% |
| | Laminin | 80% | 77% |
| HES-3 | Matrigel | 68% | 68% |
| | Laminin | 0% | 13% |

**Figure 7** Comparison of hESC growth and expression of pluripotent markers on laminin (♦, ◊) versus Matrigel (■, □) coated DE53 microcarriers in agitated spinner flask cultures. Growth kinetics and viability of HES-2 (A) and HES-3 (B) cultures. Error bars indicates standard error. (C) Percentage of cells expressing mAb84 and Tra-1-60 after 7 days in culture.

microcarriers (Fig. 1) probably as a result of masking of the positively charged or collagen attachment ligands. This phenomenon was described earlier by Mukhopadhyay et al. (1993) who shows that serum adsorption on microcarriers resulted in reduced cell attachment of Vero cells as a result of decreased surface charge. On the other hand, in a similar manner in which Matrigel coating of 2D tissue cultures plate supports long term hESC propagation (Xu et al., 2001), the coating of the microcarriers allowed for long term growth of undifferentiated hESC. Cell growth and pluripotency in these microcarrier cultures were not affected significantly by the properties of the microcarriers: These include the type of positive charge (tertiary amine (DE52 and DE53) versus quaternary, QA52), the degree of positive charging (0.88–1.08 meq/g dry materials for DE52 as compared with 1.8–2.2 meq/g for DE53), the shape and matrix of the microcarrier (Dextran spherical microcarrier (Cytodex 1) versus cellulose cylindrical microcarrier (DE53)) and the type of ligand (positively charged Cytodex 1 versus collagen coated Cytodex 3 microcarriers). We assume that Matrigel masks the different microcarrier surface properties enabling hESC to maintain their pluripotent state.

In summary we have shown that various Matrigel coated microcarriers can support long term propagation of

undifferentiated hESC. HES-2 and HES-3 were propagated for over 17 passages on Matrigel coated DE53 and Cytodex 1 microcarriers (over 11 passages). Similar results were reported by Nie et al. (2009), the authors demonstrate that by coating Cytodex 3 microcarriers with Matrigel, they were able to propagate H9 cells for at least 10 passages while expressing pluripotent markers at levels comparable to those from 2D cultures on Matrigel coated plates (Nie et al., 2009).

The size and shape of the microcarriers has an effect on the mode of propagation and cell yield. hESC grew as compact cell-microcarrier aggregates on the cylindrical shaped (L 130 μm × D 35 μm) positive charged cellulose microcarriers (DE52, DE53 and QA52) and as a less compact cell-microcarrier aggregate on the beaded 190 μm diameter Cytodex1 microcarrier (Figs. 2 and 3). These different modes of propagation did not affect cell yield and pluripotency. Reduction of the bead diameter from 190 μm (Cytodex1) to 65 μm (Tosoh65 PR) and 10 μm (Tosoh10 PR) resulted in generation of more dense cell-microcarrier aggregates (Fig. 2). In fact, the 10 μm spherical microcarriers which are smaller than the cells serve only as a linker between the cells for the generation of condensed cell-microcarrier aggregates. These tight structures led to a decrease in cell yield (Fig. 1E) probably as a result of limited access of nutrient and growth factors to the cells. Cell yields from the macroporous microcarrier cultures (Cytopore 2 and Cultispher G) were also relatively low (Fig. 1E). We assume that the macroporous beads might provide a non-uniform exposure of cells to nutrients and growth factors, whereby cells inside the pores have less access to growth factors (Nie et al., 2009; Lock and Tzanakakis, 2009). Moreover, Cultispher G cultures resulted in a decrease in pluripotency after the second passage (52–64% cells expressing Tra-1-60) probably due to the low Matrigel adsorption onto Cultispher G.

These results show that the shape and size of Matrigel coated microcarriers have an effect on aggregate formation, which in turn affected hESC growth. To investigate these effects more thoroughly, further exploration using more controlled shapes and size distributions of microcarriers is needed.

The use of laminin as an alternative substrate for Matrigel in 2D plate cultures has been reported by several groups (Xu et al., 2001; Beattie et al., 2005). In this study, we have shown that mouse laminin can also replace Matrigel in 3D microcarrier cultures. Two cell lines (HES-2 and HES-3) were propagated for long periods (10 passages) on two different laminin coated, positively charged microcarriers (Cytodex 1 and DE53). The cultured cells showed stable karyotype and retained pluripotency. hESCs were capable of differentiating into cells of the three germ layers by *in vitro* spontaneous differentiation via embryoid bodies, and teratoma formation in SCID mice. In general, similar cell yields were obtained in cultures of laminin coated DE53 microcarrier compared to Matrigel coated ones. Recently, Rodin et al. (2010) identified laminin-511 within the human laminin family as the important substrate supporting long term cultivation of undifferentiated hESC. Moreover, they showed that laminin-511 has better adhesion property than laminin-111, which is found in purified natural mouse laminin. Thus, it is possible that coating of microcarriers with human laminin-511 could improve cell yields.

We have demonstrated recently that the effect of agitation on cell differentiation is cell line specific. HES-2 cells propagated on Matrigel coated DE53 in agitated spinner flasks maintained pluripotency, while HES-3 cells tend to differentiate during propagation (Leung et al., 2010). This phenomenon was accentuated when using laminin coated microcarriers. HES-2 cells on laminin coated microcarriers showed similar expression of pluripotent markers to Matrigel coated controls. But HES-3 cultured on laminin coated microcarriers completely lost their expression of pluripotent markers (Fig. 7C). Moreover the viability of HES-3 cells propagated on laminin coated microcarriers was considerably lower than on Matrigel coated ones. These results show that the Matrigel coating may offer a shear protective element, which we assume, arises from the gelatinous polymeric nature of the thick coating. hESC propagated on a laminin-111 coated surface has lower cell contact area when compared to Matrigel, as reported by Rodin et al. (2010). Hence we postulate that under agitated conditions, the lower cell contact of HES-3 on laminin coated microcarriers might have resulted in cell detachment resulting in lower viability, differentiation and consequently a reduced final cell yield (Fig. 7C). It will be of interest to test laminin-511 which was shown to have greater cell contact area, in spinner flask cultures (Rodin et al., 2010).

In conclusion, we have shown that conventional microcarriers intended for cultivation of mammalian cell lines are not optimized for long term undifferentiated hESC cultivation. There is a need for ECM (Matrigel) or laminin coating in order to achieve long term propagation of undifferentiated cells. The mode of cell growth and the cell yield are generally not affected by the microcarrier surface properties but rather by their shape and size. Cell propagated on laminin coated carriers are extremely sensitive to shear forces. These two issues can be the targets for research into optimizing laminin coated microcarriers for hESC propagation.

## Material and methods

### Cell culture

The human embryonic stem cell line HES-2 (46 X,X) and HES-3 (46 X,X) were obtained from ES Cell International and maintained on Matrigel-coated tissue culture plate with mouse embryonic fibroblasts conditioned medium (MEF-CM) as previously described (Chin et al., 2007; Choo et al., 2006). Cell counts (total and non-viable) were measured by the nuclei count method using Nucleocounter (Chemometec).

### Preparation of microcarriers

Table 1 provides comprehensive details on the microcarriers used in this study. Spherical resins, Toyopearl AF-Tresyl-650 (mean ∅ 65 ± 25 μm (Tosoh 65)) and TSKgel Tresyl-5PW (∅ 10 μm (Tosoh 10)) were derivatized with protamine sulfate (Sigma-Aldrich, Cat no. P3369) as per manufacturer's instruction. The residual tresyl groups on the resins were then blocked with 0.1 M Tris–HCl, pH 8.0 for 1 h. Resins were washed with phosphate buffer saline (without $Ca^{2+}$ and $Mg^{2+}$)

at pH 7.2 and sterilized by gamma radiation. All other microcarriers: DE53, DE52, QA52, CM52, Cytodex 1, 3, Cultispher G and Cytopore 2 were hydrated and rinsed in phosphate buffer saline (without $Ca^{2+}$ and $Mg^{2+}$) at pH 7.2 and sterilized by autoclaving.

## Coating microcarriers with ECM components

Matrigel (BD Matrigel™ Basement Membrane Matrix Material) was obtained from BD Biosciences. Matrigel was diluted 30 times in ice cool Knockout (KO)-medium before using it as previously described in Choo et al. (2006). Microcarrier coating was carried out by adding 1 ml of the diluted Matrigel solution to the following amount of microcarriers: 5 mg of cellulose based microcarrier (DE53, DE52, QA52 and CM52), 0.6 mg of Cytopore 2, 1.25 mg of Cytodex 1 or 3, 1.25 mg of Tosoh 65 coupled with protamine, 0.13 mg of Tosoh 10 coupled with protamine, and 0.6 mg of Cultispher G. The microcarriers in Matrigel solution were agitated at 4 °C overnight and equilibrated with MEF-CM before use.

To prepare laminin coated microcarriers, 40 μg aliquot of laminin from Invitrogen (Natural mouse laminin purified from the Engelbreth-Holm-Swarm sarcoma, Cat no. 23017–015) was added to either 10 mg DE53 or 5 mg Cytodex 1 microcarriers in 1 ml phosphate buffer saline solution. The laminin coated microcarrier preparation was agitated at 4 °C overnight and equilibrated with MEF-CM before use. Similarly, 100 μg of fibronectin (Fibronectin from human plasma, Sigma-Aldrich Cat no. F0895) or 6 μg of vitronectin (Vitronectin from human plasma, Sigma-Aldrich Cat no. V8379) was coated onto the microcarriers.

To screen ECM components, 1 mg of bovine heparan sulfate (Sigma-Aldrich Cat no. H7640); 1 mg of porcine heparan sulfate (Sigma-Aldrich Cat no. H9902); 1.4–3.5 mg of bovine hyaluronic acid (Sigma-Aldrich Cat no. H7630); or 1.4–3.5 mg of hyaluronic acid from *Streptococcus* (Sigma-Aldrich Cat no. H7630) were added to 20 mg of DE53 microcarriers in 1 ml phosphate buffer saline. The microcarriers in ECM solutions were agitated at 4 °C overnight and equilibrated in MEF-CM before use. Control uncoated microcarriers were incubated in MEF-CM in 4 °C overnight.

## Cultivation of hESC on microcarriers in 6-well plates

Prior to cell seeding, ultra low attachment 6-well plate (Corning Cat no. 3471) containing microcarriers in 4 ml of MEF-CM were equilibrated for 1 h in 37 °C/5% $CO_2$ incubator. The initial seeding density was 1.6 to $2\times10^5$ cells/ml. After topping up to final volume of 5 ml, the plate was then placed on an orbital shaker at 110 rpm in 37 °C/5% $CO_2$ incubator to promote adhesion to microcarriers. Final microcarrier concentrations were 4 mg/ml for cellulose based microcarriers (DE53, DE52, QA52 and CM52), 1 mg/ml for all macroporous (Cultispher G and Cytopore 2) and spherical microcarriers (Cytodex1, Cytodex 3 and Tosoh 65 PR) and 0.1 mg/ml for Tosoh 10 PR.

The microcarrier cultures were cultivated for 7 days under static condition and 80% of the growth medium was refreshed daily. At the end of the culture, cell numbers and percentage of cells expressing pluripotent markers were assessed. To passage, after 7 days cell-microcarrier aggregates were mechanically dissociated and seeded into new 6-well plates at seeding density of $0.8–1.6\times10^5$ cells/ml. Cell concentrations were measured by the nuclei count method using Nucleocounter (Chemometec).

## Measurement of cell attachment to microcarriers and 2D cultures

A hESC single cell suspension was obtained by dissociating confluent HES-3 from a 6 cm tissue culture dish with Accutase (Invitrogen). Viable cells ($2\times10^5$ cells/ml) from the single cell suspension were seeded into 6-well ultra low attachment plate containing 5 ml MEF-CM medium and microcarriers at the concentrations given in Table 1. The cultures were maintained in static conditions in 37 °C/5% $CO_2$ incubator, the plates were agitated for 2 h on orbital shaker at 110 rpm. After 2 h more than 80% of the cells attached to positively charged microcarriers. Aliquots of supernatant were withdrawn and the number of viable unattached cells was measured. For 2D colony cultures in 6-well plates, cell attachment efficiency was measured in static conditions. The attachment efficiency is then calculated by subtracting the unattached cells from the initial viable cell concentration.

## Cultivation of hESC on microcarriers in spinner flask

Static microcarrier cultures from 6-well plates were seeded into spinner flasks. Briefly, the exponentially growing hESC microcarrier culture was mechanically dissociated into small cell clumps as previously described (Oh et al., 2009; Chen et al., 2010) and then seeded at $4\times10^5$ cells/ml in a 100 ml spinner flask (Bellco Cat. No. 1965–00100), containing 25 ml of MEF-CM and 8 mg/ml of laminin or Matrigel coated DE53 microcarriers. The culture was incubated at 37 °C/5% $CO_2$ in static condition for 24 h. The medium was then topped up to 50 ml and the culture was agitated at 25 rpm. 80% of Growth medium was replaced daily with fresh MEF-CM. Cell concentration was monitored daily and pluripotent markers were measured on day 7.

## Analyses of pluripotent markers Tra-1-60, Mab84 and Oct4

The expression levels of extracellular surface marker Tra-1-60 and Mab84 (Choo et al., 2008) in hESC populations were monitored by fluorescent flow cytometry as described previously (Oh et al., 2009).

Immunohistochemical staining of Tra-1-60 and Oct4 on the microcarrier culture was carried out as described previously (Oh et al., 2009). Alexa-fluor® 488 and 594-conjugated $F(ab')_2$ fragment of goat anti-mouse IgG (Invitrogen) were used as secondary antibodies.

## Differentiation study

Spontaneous differentiation of hESC microcarrier cultures was carried out *in vitro* by embryoid body (EB) formation according to Chin et al. (2007). Briefly, after 7 days of

**Table 2** Primer sequences used for quantitative RT-PCR.

| Gene name | Primers used for quantitative real-time PCR |
|---|---|
| OCT4 | F: 5′-CTGCAGCAGATCAGCCACAT-3′ |
| | R: 5′-TCGGACCACATCCTTCTCG-3′ |
| NANOG | F: 5′-ACCAGAACTGTGTTCTCTTCCACC-3′ |
| | R: 5′-CCATTGCTATTCTTCGGCCAGTTG-3′ |
| AFP | F: 5′-TCCCTCCTGCATTCTCTGATG-3′ |
| | R: 5′-CCTGAGCTTGGCACAGATCC-3′ |
| GATA6 | F: 5′-GCGGCTTGGATTGTCCTGT-3′ |
| | R: 5′-TGCGCCATAAGGTGGTAGTTG-3′ |
| Hand 1 | F: 5′-CCACCCTTTTGGAGCGAATT-3′ |
| | R: 5′-AATTAGAGAAGACGGCGTCGG-3′ |
| Nkx2.5 | F: 5′-TCCCCTGGATTTTGCATTCA-3′ |
| | R: 5′-AGGATCACTCATTGCACGCTG-3′ |
| Pax6 | F: 5′-CCAGCTTCACCATGGCAAAT-3′ |
| | R: 5′-GGCAGCATGCAGGAGTATGAG-3′ |
| Lamb 1 | F: 5′-CTGCAAGGATCTGTCAATGCC-3′ |
| | R: 5′-CGAGCATACACTCCCTGGAAA-3′ |
| GAPDH | F: 5′-GTCGGAGTCAACGGATTTGG-3′ |
| | R: 5′-AAAAGCAGCCCTGGTGACC-3′ |

differentiation the mechanically dissociated EBs were re-plated onto gelatin-coated 6-cm tissue culture plate and then cultured for another 14 days.

RNA from the differentiated cells was harvested using RNA extraction kit from Qiagen (RNeasy Mini Kit, cat no. 74104) with DNase treatment. cDNA was synthesized using Superscript II Reverse Transcriptase (Invitrogen) for subsequent quantitative RT-PCR containing Power SYBR Green PCR Master Mix (Applied Biosystems) with primers of genes listed in Table 2. PCR was carried out in ABI Prism7000 Sequence Detection System (Applied Biosystems) using the following amplification parameters: 2 min at 50 °C, 10 min at 95 °C, and 40 cycles of 15 s at 95 °C, followed by 1 min at 60 °C. The relative Cycle Threshold (Ct) was determined and normalized against the endogenous GAPDH gene. The fold change of each gene was compared against the same gene prior to differentiation.

Immuno-staining was carried out according to Chan et al. (2008) to identify cells from the three embryonic germ layers. Briefly, differentiated hESC were fixed with 4% paraformaldehyde for 15 min and blocked for 2 h in PBS buffer containing 0.1% Triton X-100, 10% goat serum and 1%BSA. The primary antibody was diluted in 1%BSA/PBS at the following concentrations: 1:400 for α-smooth muscle actin (SMA) (Sigma-Aldrich), 1:1000 for β-III Tubulin (Sigma-Aldrich) and 1:250 for α-fetoprotein (AFP) (Sigma-Aldrich). Cells were then washed in 1% BSA/PBS and incubated in the dark with FITC-conjugated secondary antibodies for 2 h at room temperature. After another wash with 1%BSA/PBS, fluorescent mounting medium with DAPI (Vectashield Cat no. H-1200) was added to cover the cells and incubated 1 h before immuno-fluorescence was visualized and captured using Zeiss Axiovert 200 M fluorescence microscope (Carl Zeiss).

For *in vivo* differentiation, mechanically dissociated hESC cell-microcarrier aggregates were plated onto Matrigel-coated tissue culture plate. After 7 days, cells were mechanically harvested using Invitrogen STEMPRO® EZPassage™ Tool. About 4 to $5\times10^6$ cells were injected into SCID mouse as described previously (Choo et al., 2006). The tumor was dissected, embedded in paraffin, sectioned and stained with hematoxylin-eosin for histological examination.

## Scanning electron microscopy

The microcarrier-cell aggregates from 6-well plates were washed 3 times in sterile PBS with $Ca^{2+}$ and $Mg^{2+}$ and fixed in 3% glutaraldehyde/1% paraformaldehyde/PBS and followed by washing three times with PBS. The microcarrier-cell aggregates were then dehydrated using increasing ethanol concentration (25%, 50%, 75% then 100%) with incubation time of 30 min at each step. The dehydrated samples were deposited into microporous specimen capsules (>100 μm) followed by critical point drying (Critical Point Dryer CPD 030, BAL-TEC AG). Afterward the samples were then deposit on self adhesive carbon tape and mounted on aluminum stubs. Samples were analyzed with a JSM-6390LV scanning electron microscope (JEOL Ltd).

## Karyotype analysis

hESC from passage 10 of laminin-coated microcarrier cultures were harvested and sent for karyotype analysis, as described previously (Oh et al., 2009). Karyotype analysis was performed with 20 cells.

## Statistical analysis

Figures show standard errors representing at least three measurements. Student's t-tests were carried out to determine the significance between different experimental conditions ($p<0.01$ is considered as significant).

Supplementary materials related to this article can be found online at doi:10.1016/j.scr.2011.04.007.

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